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Embryos of *Homarus americanus* are Protected by Epibiotic Bacteria

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Abstract. Embryos of the American lobster, *Homarus americanus,* are remarkably resistant to infection by the fungus *Lagenidium callinectes,* a pathogen of many crustaceans. The surfaces of healthy lobster embryos are covered almost exclusively by a single, Gram-negative bacterium, which grows in a dense mosaic pattern. In culture, this bacterium produces a compound that completely inhibits the growth of the pathogenic fungus *in vitro* at 10 mcg/ml. Large-scale fermentation, extraction, and subsequent chromatographic purification led to the identification of the antifungal substance as 4-hydroxyphenethyl alcohol (tyrosol), an antibiotic substance known to be produced by terrestrial fungi.

Introduction

Like several other decapod crustaceans, the American lobster *Homarus americanus* incubates its embryos externally, and each female carries a large cluster comprising up to 60,000 embryos. The embryos are attached to specialized abdominal pleopods until hatching some nine months after fertilization (Cobb and Wang, 1985). Throughout this long brooding period, during which the female is said to be "in berry," the embryos are continuously exposed to water-borne microorganisms. It is remarkable that the seemingly unprotected embryos can survive microbial encroachment. The phycomycetous fungus *Lagenidium callinectes* is a pathogen of many crustaceans. Larvae and juveniles of the American lobster, when kept in unnatural conditions, *e.g.,* in aquaria, are extremely vulnerable to infection by this fungus (Fisher *et al.,* 1976; Nilson *et al.,* 1976; Provenzano, 1985). In contrast, *Homarus* embryos, even when detached from the female, appear to be remarkably resistant to fungal attack. This situation is highly analogous to that recently observed for the estuarine shrimp *Palaemon macrodactylus* (Gil-Turnes *et al.,* 1989). Embryos of *P. macrodactylus* were also impervious to attack by *L. callinectes.* In that study, *Palaemon* embryos were found to host an epibiotic bacterium that produced 2,3-indolenedione, a molecule toxic to *L. callinectes.* The intent of this research was to compare *Homarus americanus* with *Palaemon macrodactylus,* and to determine whether lobster embryos are also protected by an association with symbiotic bacteria.

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Materials and Methods

Embryo infection experiment with Lagenidium callinectes

Gravid female *Homarus americanus* were collected in the vicinity of Martha's Vineyard, Massachusetts, air shipped to California, and maintained in aquaria at the Bodega Marine Laboratory in Bodega Bay, California (UC-Davis). The embryos were observed to be at different stages of development. Ten groups of five embryo clusters each were detached from the females. Each cluster was rinsed with three aliquots of sterilized seawater and subsequently suspended from a cotton thread in aerated 125 ml Erlenmeyer flasks containing 75 ml of sterile seawater. A liquid suspension of *Lagenidium callinectes* was prepared by homogenizing a 0.5 cm diameter agar core of fungal hyphae in 10 ml of 2216 Difco Marine Broth medium. The fungus grew for one week, and the culture was then shaken vigorously to break up the hyphae. Aliquots of 1 ml of this thick suspension were added to each experimental flask. Addition of fungal culture was repeated after the fifth day on a daily basis for a period of two weeks.

Isolation and culture of associated bacteria

In a typical experiment, five embryos from each animal were homogenized in an autoclaved tissue grinder with 10 ml of sterile seawater. One drop of the homogenate, and of 1/10 and 1/100 dilutions, were plated on Difco 2216 Marine Agar plates. Colonies were removed and subcultured after 1–3 weeks. Although three or four morphologically variable colonies were generally observed, one distinct bacterium (SGT-76), a salmon-colored, slow-growing (at 21°C), Gram-negative rod, was consistently obtained. In liquid culture, this strain inhibited the growth of *L. callinectes*. Antifungal testing was performed by cutting agar cores, 0.5 cm in diameter, from lawns of the pure bacterium and placing them on agar plates approximately 1 cm from agar cores containing radiating hyphae of *L. callinectes*. Because of the inhibition observed, this bacterium was selected for subsequent chemical studies.

Extraction and purification of the antifungal compound

The Gram-negative, salmon-colored bacterium, SGT-76, was cultured, at 21°C, in a 16-l carboy using a medium composed of 3 g BactoPeptone (Difco) and 5 g yeast extract per liter of seawater. The culture grew, with aeration, for three weeks. The final pH of the medium was 8.8. The entire culture was extracted twice with 4 l ethyl acetate. After evaporation of the combined solvents, the remaining crude extract was fractionated by silica-gel vacuum flash chromatography using variable amounts of ethyl acetate in isooctane. The antifungal activity of each fraction was determined by placing 0.5 mg of each dry fraction onto a 0.5 cm paper disk and placing the disk at the edge of fungal growth. The active compound eluted with 80% ethyl acetate/isooctane. Final purification of the antifungal compound was achieved by size exclusion chromatography on Sephadex LH20 using a mixture of hexane/methylene chloride/methanol (2:5:1). The purified compound was characterized by infrared spectroscopy (IR), by high-resolution mass (HRMS) and by ^1H and ^{13}C nuclear magnetic resonance spectrometry (NMR).

Scanning electron microscopy

Embryos were fixed in 2.5% glutaraldehyde in 3% saline solution for a minimum of 24 h. After three rinses in 3% saline solution [illegible] min, each specimen was transferred to a solution of [illegible]um tetroxide in 3% saline solution for nine minutes. Specimens were then stored in saline solution overnight and subsequently dehydrated using an acetone/distilled water sequence: 35% for 15 min, 50% for 15 min, 75% for 30 min, 95% for 1 h, and 100% ethanol for 12 h. Critical point drying was done under CO_2 and the gold coating thickness was 300 Å. Electron micrographs were obtained with a Hitachi Model 539 SEM. Pure bacterial films were prepared as follows: a drop of a 3-day liquid culture was deposited on a small millipore filter (0.25 μ pore size) placed on an agar plate. As soon as growth was visible, the specimens were fixed in 3% formaldehyde and 3% glutaraldehyde in 0.2 *M* sodium cacodylate trihydrate buffer (pH 7.4) for 1 h and then washed three times for 5 min in 0.2 *M* cacodylate solution. The specimens were then transferred to 2% osmium tetroxide in 0.2 *M* cacodylate solution for 1 h, and subsequently rinsed six times for 5 min in 0.2 *M* cacodylate solution. After dehydration using a sequence of ethanol/distilled water treatments for 10 min each, the specimens were critical-point dried under CO_2, coated with gold (300 Å), and micrographs obtained with a Hitachi Model S450A SEM.

Results

After 18 days and ten additions of fungal culture, *H. americanus* embryos appeared healthy and free of fungal infection. The visible organ anatomies and heartbeat rates of the treated embryos were identical to those of the controls. Scanning electron micrographs showed that the embryonal surface was covered by an almost monoculture of a rod-shaped bacterial strain (Fig. 1A). Some of the embryos, at different stages of development, were occasionally found to have very sparse coverage by three morphologically different bacteria (Fig. 1B), in addition to the rod-shaped strain. Older embryos, near hatching, were consistently observed to possess dense coverage by an almost monoculture of the rod-shaped bacterium (Fig. 1C).

Replicate inoculations of embryo homogenates on Marine Agar plates resulted in the isolation of a maximum of four, but usually fewer, strains of bacteria. One of the strains (SGT-76), which was consistently isolated, was inhibitory to *L. callinectes*. This bacterium, a Gram-negative rod insensitive to penicillin, was a pale salmon-colored strain, and it was extremely slow growing on agar plates and in liquid medium. For reasons unknown, the pH of the culture medium seemed to rise to 8 or more during fermentation. This rise in pH could provide a possible explanation for the poor growth observed. Scanning electron micrographs of this bacterium, grown on millipore filters, showed that the cells were identical in size and shape, and had an identical growth pattern to those observed on the surface of the natural embryos (Fig. 1D).

An antifungal compound produced by the bacterial strain grown in liquid medium was isolated and identified as tyrosol, 4-hydroxyphenethyl alcohol (Fig. 2). The active compound was isolated as a viscous oil which showed the following spectral characteristics: IR (film): 3400, 3150 cm^{-1}; HRMS requires 138.04 for $C_8H_{10}O_2$, found 136.06; ^1H NMR, 200 MHz (acetone-d_6): 7.0 (d, 2 H, J = 8.6 Hz), 6.6 (d, 2 H, J = 8.6 Hz), 3.7 (t, 2 H), 2.7 (t, 2 H);

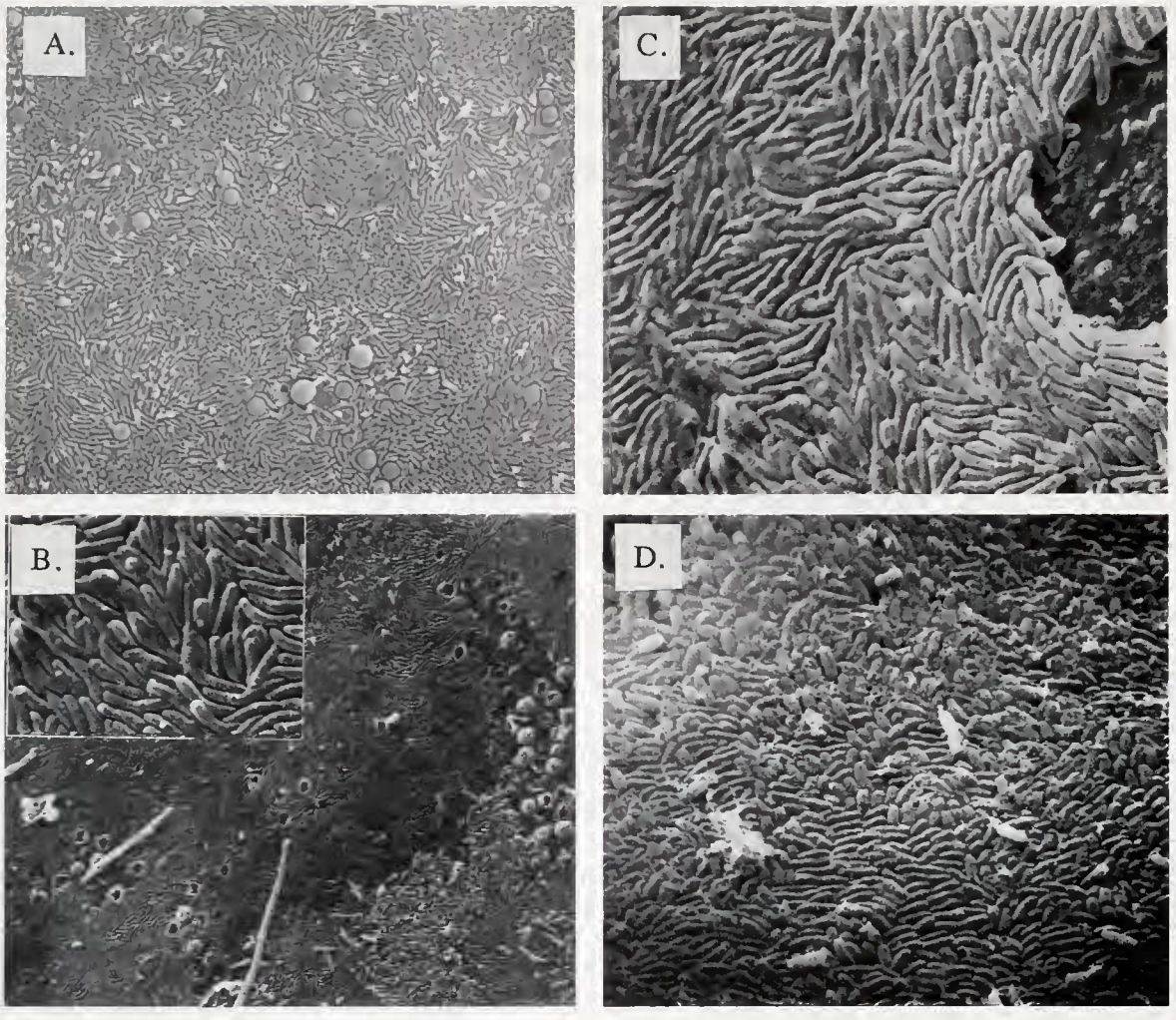
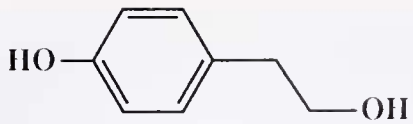


Figure 1. Scanning electron micrographs of healthy embryos of *Homarus americanus* under various conditions. (A) Surface of embryo after exposure to the fungus *Lagenidium callinectes*, illustrating the lack of fungal attachment (1250×). (B) Surface of embryo showing the coverage by the colonial rod morphotype and the other three types occasionally found (1000×). (C) Surface of embryo, at near full gestation, showing extensive and thick coverage by the rod-type bacterium (5000×). (D) Micrograph of the pure bacterium grown on a millipore filter (2000×).

^{13}C NMR, 200 MHz (acetone-d_6): 155.0, 131.0, 130.0, 115.5, 115.4, 63.8, 39.1 (Fig. 2). All chemical and spectral data were identical to those from the commercially-available 4-hydroxyphenethyl alcohol (tyrosol, Aldrich #18,825-5). Tyrosol effectively inhibited growth of *L. callinectes* in liquid culture at a concentration of 10 mcg/ml. In agar plate assays, 100 mcg tyrosol per disk resulted in an 8 mm zone of fungal inhibition.

Discussion

All the embryos observed were covered largely by a single, rod-shaped bacterium, distinguishable from other types by its characteristic dense, mosaic-like growth pattern. The oldest embryos had the thickest coverage by this particular strain, thus health and successful development appear to be related to the degree of bacterial coverage. Harper and Talbot (1984), who investigated embryos of several *Homarus* species to determine if the presence of epibiotic bacterial flora was related to loss of embryos from the pleopods, also observed four bacterial morphotypes. Their bacteria appear to be morphologically identical to those described in this article, including one that they described as a "colonial rod." They found that embryos from wild born and wild spawned *H. americanus* were heavily covered by bacterial rods, and that these embryos were successfully retained by the adult until hatching. Based upon the repetitive isolation of the salmon-colored bacterium (SGT-76) from healthy embryos, and its highly characteristic mosaic growth pattern on natural



4-hydroxyphenethyl alcohol

"Tyrosol"

Figure 2. Chemical structure of the antifungal metabolite 4-hydroxyphenethyl alcohol.

surfaces and on filters, we believe that this bacterium is the natural epibiont of *Homarus* embryos. At the same time, we recognize that this proposal will be difficult to rigorously prove.

The antifungal compound produced by bacterium SGT-76, 4-hydroxyphenethyl alcohol, or tyrosol, has previously been reported as a natural product from two fungal species that are involved in symbiotic associations with plants (Stoessl, 1969; Claydon *et al.*, 1985). In an apparently similar adaptation, those fungi seem to protect their hosts against invasion by pathogenic fungi.

Protection of embryos by epibiotic bacteria has been shown previously (Fisher, 1983; Gil-Turnes *et al.*, 1989) for the caridean shrimp *Palaemon macrodactylus*, also an external brooder. In the present study, the association of *H. americanus* embryos with a Gram-negative, rod-shaped bacterium suggests a similar adaptation in which a vulnerable host is protected against pathogenic microorganisms by symbiotic bacteria. At least one explanation for the resistance of *Homarus* embryos is the bacterial production of the antifungal compound tyrosol in nature. Although tyrosol is only a moderately potent antifungal agent, the dense bacterial coverage observed would easily result in high levels of the compound at the embryo surface. Thus, tyrosol could function effectively to reduce fungal encroachment.

The antifungal agents isolated from crustacean-associated bacteria to date (tyrosol and 2,3-indolinedione from a *Palaemon macrodactylus*-associated bacterium), are simple molecules with only modest potencies. These molecules appear to be unusually effective against *Lagenidium*, however, perhaps suggesting that they are targeted to this and related fungal pathogens.

Studies of the bacterial symbionts of commercially important marine invertebrates could provide important information leading to the control of disease under aquaculture conditions. Indeed, there is a significant need to develop inexpensive and environmentally safe antifungal agents for this specific application. The simple molecules discussed here, which appear to be derived from the common amino acids tyrosine and tryptophane, should be considered in this application.

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Literature Cited

Claydon, N., J. F. Grove, and M. Pople. 1985. Elm bark beetle boring and feeding deterrents from *Phomopsis oblonga*. *Phytochemistry* **24**(5): 937–943.

Cobb, J. S., and D. Wang. 1985. Fisheries biology of lobsters and crayfishes. Pp. 168–248 in *The Biology of Crustacea*. Vol. 10, Academic Press, New York.

Fisher, W. S. 1983. Eggs of *Palaemon macrodactylus*: II. Association with aquatic bacteria. *Biol. Bull.* **164:** 201–213.

Fisher, W. S., E. H. Nilson, L. F. N. Follett, and R. A. Shleser. 1976. Hatching and rearing lobster larvae (*Homarus americanus*) in a disease situation. *Aquaculture* **7:** 75–80.

Gil-Turnes, M. S., M. E. Hay, and W. Fenical. 1989. Symbiotic marine bacteria chemically defend crustacean embryos from a pathogenic fungus. *Science* **246:** 117–118.

Harper, R. E., and P. Talbot. 1984. Analysis of the epibiotic bacteria of lobster (*Homarus*) eggs and their influence on the loss of eggs from the pleopods. *Aquaculture* **36:** 9–26.

Nilson, E. H., W. S. Fisher, and R. A. Shleser. 1976. A new mycosis of larval lobster (*Homarus americanus*). *J. Invertebr. Pathol.* **27:** 177–183.

Provenzano, A. J., Jr. 1985. Culture of crustaceans: general principals. Pp. 279–280 in *The Biology of Crustacea*, Vol. 10, Economic Aspects: Fisheries Culture. Academic Press, New York.

Stoessl, A. 1969. 8-Hydroxy-6-methoxy-3-methylisocoumarin and other metabolites of *Ceratocystis fimbriata*. *Biochem. Biophys. Res. Comm.* **35**(2): 186–191.